# Specificity of the Interaction between the *Paracoccus denitrificans* Oxidase and Its Substrate Cytochrome *c*: Comparing the Mitochondrial to the Homologous Bacterial Cytochrome $c_{552}$, and Its Truncated and Site-Directed Mutants†

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ABSTRACT: Under in vitro conditions, bacterial cytochrome *c* oxidases may accept several nonhomologous *c*-type electron donors, including the evolutionarily related mitochondrial cytochrome *c*. Several lines of evidence suggest that in intact membranes the heme $aa_3$ oxidase from *Paracoccus denitrificans* receives its electrons from the membrane-bound cytochrome $c_{552}$. Both the structures of the oxidase and of a heterologously expressed, soluble fragment of the $c_{552}$ have been determined recently, but no direct structural information about a static cocomplex is available. Here, we analyze the kinetic properties of the isolated oxidase with the full-size $c_{552}$, with two truncated soluble forms, and with a set of site-specific mutants within the presumed docking site of the cytochrome, all heterologously expressed in *Escherichia coli*. Our data indicate that all three forms, the wild type and both truncations, are fully competent kinetically and exhibit biphasic kinetic behavior, however, under widely different ionic strength conditions. When mutations in lysine residues clustered around the interaction domain were introduced into the smallest fragment of $c_{552}$, both kinetic parameters, $K_M$ and $k_{cat}$, were drastically influenced. On the other hand, when the nonmutated truncated form was used to donate electrons to a set of oxidase mutants with replacements clustered along the docking site on subunit II, we observe distinct differences when comparing the kinetic properties of the widely used horse heart cytochrome *c* with those of the bacterial $c_{552}$. We conclude that the specific docking sites for the two types of cytochromes differ to some extent.

Essential components of the mitochondrial electron transport chain have their counterparts in many bacterial respiratory complexes, often with a much simpler subunit structure, but virtually identical in their basic functional properties. This is exemplified by the cytochrome *c* oxidase of the bacterium *Paracoccus denitrificans*, composed of only four different subunits; when its three-dimensional (3D) structure (*1*, *2*) is compared to that of the mitochondrial oxidase (*3*), it is evident that the three largest subunits (encoded by the mitochondrial genome) of the eukaryotic enzyme are homologues of the bacterial enzyme and represent the catalytic core in the mitochondrial enzyme. The mammalian oxidase, besides being a dimer in structure, is composed of a further 10 subunits which are nuclear-encoded and to which regulatory properties have been attributed (for a review, see ref *4*). Being also easily amenable to mutagenesis, bacterial respiratory complexes thus serve as suitable model systems for the study of processes of electron transfer and energy transduction mechanisms (*5*−*8*).

Electrons from cytochrome *c* are first transferred to the homobinuclear copper center, $Cu_A$, located within 5 Å of the surface on the periplasmically (or the mitochondrial intermembrane space) oriented hydrophilic domain of subunit II. Interaction with the donor protein cytochrome *c* has long been ascribed to charged residues mainly (*9*−*11*), with the putative docking site on subunit II providing exposed acidic residues, whereas the mitochondrial cytochrome *c* is strongly positively charged on its interaction face (*12*). More recent studies have extended this view to include some residues on subunits I and III contributing to the docking site as well, and to point at a patch of hydrophobic residues in mediating the fine-tuning of the protein interaction for efficient electron transfer (*13*−*16*). A surface-exposed tryptophan residue in subunit II (W121 in the *Paracoccus* numbering system, used throughout here) plays a crucial role as a primary acceptor in *Paracoccus* and related bacterial oxidases as shown by site-directed mutagenesis (*16*, *17*; see also Figure 5).

When the purified bacterial enzymes were assayed, horse heart cytochrome *c* was used since often the bacterial homologous electron donors are not identified with certainty, or are in short supply. In the *P. denitrificans* case, a large number of potential candidates have been identified (*7*, *18*), but previous studies suggested that, contrary to the situation

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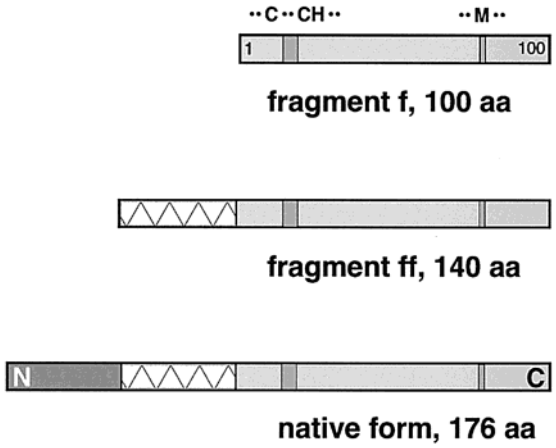


FIGURE 1: Domain structure of the *P. denitrificans* cytochrome $c_{552}$ and truncated fragments expressed in *E. coli*. Size representation (in the number of amino acids) of the soluble cytochrome $c_{552}$ fragments f (functional domain) and ff (functional and flexible domain; hatched area) and of the native membrane-bound protein with its N-terminal membrane anchor (bottom). The heme attachment motif is denoted by its amino acid ligands (top).

in mitochondria, a membrane-bound cytochrome *c* acts as the electron mediator between the $bc_1$ complex and oxidase. First isolated in a cocomplex with both its redox partners (*19*), this cytochrome $c_{552}$ was subsequently characterized for its functional role in a deletion strain and by immunological methods (*20*). More recently, the 3D structure of a heterologously expressed hydrophilic fragment of the protein comprising the core heme domain (ref *21* and see below) has been determined by X-ray and NMR techniques (*22*−*24*). Even though its isoelectric point is well below 7, its structure reveals characteristic features of a class I *c*-type cytochrome (*22*), and a putative interaction face with a clustering of lysine residues on one hemisphere of the molecule (see also Figure 3).

Here, we compare the interaction of several molecular forms, and of site-specific mutants, of this cytochrome with the isolated wild-type oxidase to conclude that it is a competent electron donor, yielding kinetic properties matching those of the previously used mitochondrial cytochrome *c*, if assayed under its appropriate ionic strength conditions. On probing the smallest fragment of this cytochrome *c* with oxidases mutated in residues along the presumed docking site, we are able to specifically map interaction sites and differentiate between both kinds of donor molecules.

## MATERIALS AND METHODS

*Site-Directed Mutagenesis and Expression of the $c_{552}$ Protein Variants.* Cloning and transformation steps were performed as described in ref *21*. Both truncated forms of the *cyc*M gene (see Figure 1) were inserted into the pET-22b(+) vector, providing a *pel*B leader sequence for translocation into the periplasm. The full size *cyc*M gene was cloned into the same vector with the addition of a C-terminal six-histidine tag. Site-directed mutagenesis on the cytochrome $c_{552}$-f[1] was performed according to the "Quick-Change" protocol (Stratagene, Amsterdam, The Netherlands). Soluble fragments ($c_{552}$-f and -ff) were purified as described previously (*21*). The membrane-bound cytochrome $c_{552}$ was isolated from cells grown at 37 °C to an $OD_{600}$ of 3 without induction by IPTG; harvested cells were resuspended in a solution of 50 mM $KP_i$ and 150 mM NaCl (pH 8.0) and broken by being passed twice through a Manton-Gaulin press (APV Schröder, Lübeck, Germany). After ultracentrifugation (100000*g* at 4 °C for 1 h), membranes were resuspended in 50 mM $KP_i$ and 150 mM NaCl (pH 8.0) and solubilized with *n*-dodecyl $\beta$-D-maltoside (1.5 g of detergent per gram of membrane protein). The supernatant was loaded on a metal affinity column (Cu-IMAC; Pharmacia) equilibrated with 50 mM $KP_i$, 20 mM imidazole, 150 mM NaCl, and 0.5 g/L dodecyl maltoside and eluted with a linear gradient of 20 to 200 mM imidazole in the same buffer. Reddish fractions were loaded on a Q-Sepharose column equilibrated with 50 mM $KP_i$, 50 mM NaCl, and 0.2 g/L dodecyl maltoside and eluted with a linear gradient of 50 to 600 mM NaCl. The cytochrome fractions were analyzed by SDS−PAGE and difference spectroscopy, concentrated, and stored at −80 °C.

*Site-Directed Mutagenesis on Subunit II of Oxidase. P. denitrificans* ST4, deleted in the *cta* operon (*25*), was complemented *in trans* with a broad host range plasmid carrying the mutated *cta* operon (*26*). Mutations were introduced by the "altered sites" protocol (Promega, Heidelberg, Germany) as described previously (*27*). Membranes were isolated and solubilized by dodecyl maltoside (*26*); cytochrome *c* oxidase was purified as described in ref *28*. Assembly and cofactor integrity of the four-subunit (mutant) oxidase complexes were routinely confirmed by SDS gel electrophoresis and by redox and ligand binding spectra, and in selected cases by total-reflection X-ray fluorescence spectrometry and redox titrations as reported previously (*25*−*27*, *29*−*31*).

*Steady-State Kinetics and Determination of the Ionic Strength Dependence.* Activity of cytochrome oxidase was measured with a Kontron Uvikon 941 spectrophotometer at 25 °C in 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.2 g/L dodecyl maltoside. The different ionic strength conditions were adjusted by adding KCl. The cytochrome $c_{552}$ concentration was varied between 0.5 and 40 $\mu$M, and the oxidation of reduced cytochrome $c_{552}$ was followed at 551 nm after adding the purified enzyme (from 40 to 400 pM). Each point in the kinetic diagram (e.g., Figure 2) is the mean of four or five individual determinations, and linear regression was used to derive the kinetic parameters. Determination of the ionic strength dependence was performed in the same buffer at 20 $\mu$M cytochrome $c_{552}$, with the ionic strength adjusted to between 1.8 and 296 mM by the addition of KCl. The buffer for an ionic strength of 1.8 mM was 2.5 mM Tris-HCl (pH 7.5) and 0.2 g/L dodecyl maltoside.

## RESULTS

*Bacterial Cytochrome $c_{552}$ Competently Donates Electrons to the P. denitrificans Oxidase.* The nonhomologous electron donor, cytochrome *c* from horse heart mitochondria, has frequently been used in the past for assays of oxidases isolated from various bacteria. Among several candidates, the membrane-bound $c_{552}$ has been suggested as the genuine electron transfer mediator in *P. denitrificans* (see the introductory section).

[1] Abbreviations: $c_{552}$-f, cytochrome $c_{552}$, soluble C-terminal 100-amino acid fragment comprising the heme domain; $c_{552}$-ff, cytochrome $c_{552}$, soluble C-terminal 140-amino acid fragment (see also Figure 1); IPTG, isopropyl thiogalactoside; IMAC, immobilized metal affinity chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; I, ionic strength.

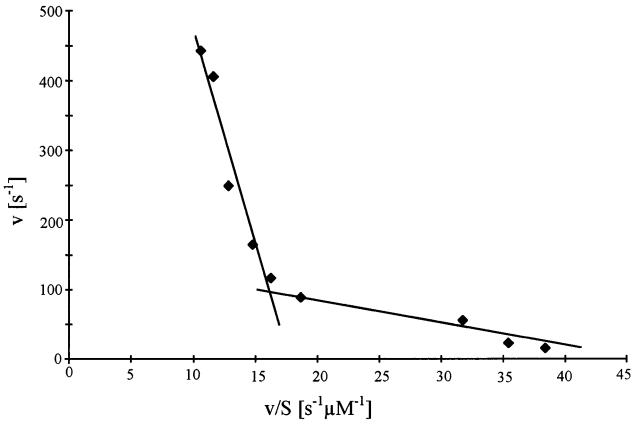


FIGURE 2: Reaction of cytochrome $c_{552}$-f with wild-type cytochrome *c* oxidase under steady-state conditions. Kinetic analysis was performed with the spectroscopic assay at 25 °C, and the results were plotted according to the Eadie−Hofstee method.

Table 1: Parameters for Both Kinetic Phases for Steady-State Turnover between Isolated Wild-Type *P. denitrificans* Oxidase Reacting with Three $c_{552}$ Modules and Horse Heart Cytochrome *c* under Different Ionic Strength and pH Conditions

| | | | high-affinity | | low-affinity | |
|---|---|---|---|---|---|---|
| cytochrome species | ionic strength (mM) | pH | $K_M$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $K_M$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) |
| $c_{552}$-f | 14.8 | 6.1 | 18 | 333 | 43.5 | 588 |
| $c_{552}$-ff | 14.8 | 6.1[a] | 1.6 | 19 | 27.0 | 137 |
| $c_{552}$ (full-length) | 14.8 | 6.1[a] | 0.8 | 23.5 | 17.5 | 270 |
| horse heart *c* | 56 | 7.5[a] | 5.9 | 363 | 14.9 | 1031 |
| $c_{552}$-f | 14.8 | 7.5[a] | 3.2 | 136 | 18.3 | 1000 |

[a] Reaction condition optimum.

Three molecular forms of this cytochrome are now available (Figure 1), a soluble fragment comprising the 100 C-terminal amino acids representing the heme domain ($c_{552}$-f), a fragment with an additional 40 amino acids specifying a putative linker region of high polarity (*20*), and the wild-type protein with its additional membrane anchor at the N-terminus, and a C-terminal histidine tag located at the opposite side of the heme crevice. Each of these three modules was expressed in *Escherichia coli* using the cytochrome *c* maturation gene cluster (*21*, *32*) for efficient heme insertion. Expression rates were around 6 mg/L for the full-size species, 5 mg/L for $c_{552}$-ff, and 14 mg/L for $c_{552}$-f.

Each of the three different cytochrome $c_{552}$ proteins was probed kinetically for its electron donor properties using isolated wild-type *Paracoccus* oxidase in steady-state turnover in the spectrophotometric assay. Under most conditions, biphasic kinetics were obtained (see below); Table 1 lists the kinetic parameters ($K_M$ and $k_{cat}$ from both phases of the reaction). Most notably, pH conditions were strikingly different for optimal turnover: the shortest module $c_{552}$-f exhibited maximal reaction at pH 7.5, as did the horse heart cytochrome *c*, whereas conditions for both the longer soluble module ($c_{552}$-ff) and the native protein containing the membrane anchor were downshifted in their pH to 6.1.

Another crucial difference in kinetic behavior is displayed in the ionic strength dependence of the reaction. As a typical feature of cytochrome *c* interactions observed in many cases (*33*, *34*), bell-shaped curves for turnover numbers versus ionic strength are also obtained for bacterial oxidases, and explained in terms of electrostatic interactions between both reactants (*17*, *27*). The strongly charged mitochondrial cytochrome *c* yields an optimal reaction with the *P. denitrificans* oxidase at an ionic strength of 56 mM (*27*, *31*), whereas this value is significantly lowered to 15 mM for all three bacterial $c_{552}$ modules (see Table 1).

These kinetic data allow the unequivocal conclusion that, if compared under optimal buffer conditions, both sets of kinetic parameters are comparable for the two strongly differing substrate proteins of mitochondrial and bacterial origins. With nonlinear kinetics observed for most of the experimental conditions (e.g., Figure 2; see also refs *27* and *31*), we focus in particular on two of the four parameters; $K_M$ is taken from the high-affinity phase and $k_{cat}$ derived from the low-affinity phase. For both these cytochrome *c* substrates, $K_M$ values fall into the low micromolar range (3.2 $\mu$M for $c_{552}$-f and 5.9 $\mu$M for the mitochondrial donor), and $k_{cat}$ values approach 1000 $s^{-1}$ for both, likewise. Interestingly, the catalytic competence is similar also for the full-length $c_{552}$ under in vitro assay conditions: due to a more favorable Michaelis constant (high-affinity phase), the $k_{cat}/K_M$ ratio (again, taken from separate phases of the reaction as discussed above; Table 1) yields virtually identical values when compared to those for the small soluble module and, to a lesser extent, also to those for the mitochondrial donor.

*The Overall Kinetic Behavior of the Different Forms of the Bacterial Electron Donor Resembles That of the Mitochondrial Cytochrome c.* The detailled kinetic analysis over a wide range of ionic strengths and substrate concentrations not only displays a strong dependence on the former (see above) but also leaves no doubt that under specified conditions, the bacterial donor in all forms tested here may elicit biphasic kinetics. As exemplified in Figure 2 in a reaction of reduced $c_{552}$-f with wild-type oxidase, two clearly separated phases show up in an Eadie−Hofstee plot. This fact has been observed consistently in the past (see the Discussion) for the mitochondrial donor protein, and may now be extended to the homologous *Paracoccus* donor. None of the different forms studied here appears to be strictly monophasic; i.e., for all substrate variations, conditions for biphasicity can be obtained by simply decreasing the ionic strength of the assay buffer (see also Table 1).

*Specific Lysine Mutants of $c_{552}$ Exhibit Increased $K_M$ as Well as Decreased $k_{cat}$ Values.* The $c_{552}$-f fragment resembles typical class I *c* cytochromes not only in size but also in its general surface charge distribution. With its structure determined both from 3D crystals (*22*) and in solution (*23*), it displays a pattern of surface-exposed lysine residues around the heme crevice on the one hemisphere that is supposed to interact with several partner proteins (Figure 3), despite the fact that it carries a slight negative net charge [calculated pI of 6.3 (*21*)] at neutral pH. Eight positions were exchanged by site-directed mutagenesis mostly to apolar side chains, and the resulting proteins were assayed for enzyme kinetics with isolated wild-type oxidase. All mutant proteins (Table 2) uniformly show a 3−5-fold increase in $K_M$ (e.g., high-affinity phase), but also a concomitant loss in $k_{cat}$ by a similar factor. It should be noted, however, that each of the single-lysine replacement proteins reacts very sensitively toward the ionic strength of the medium; i.e., mutant proteins exhibit optima in part considerably (down to 1.8 mM) below the 15 mM value determined for the wild type (details not given). Although under those conditions $k_{cat}$ values are higher, we chose to compare mutant data at a single experimental ionic

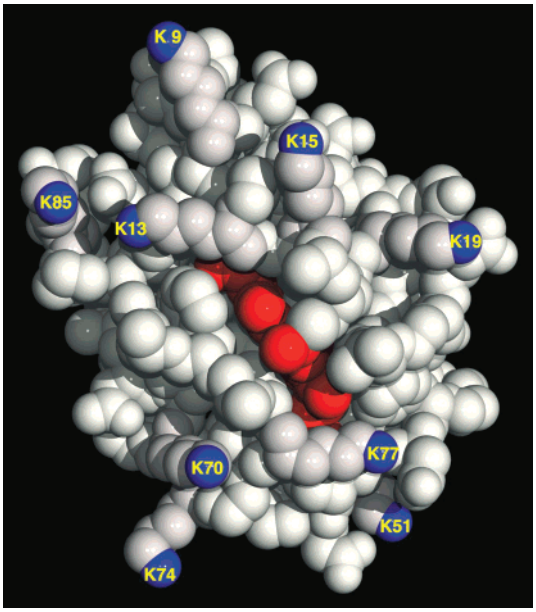


FIGURE 3: Surface-exposed lysine residues surrounding the heme crevice of the soluble cytochrome $c_{552}$-f from *P. denitrificans*. The figure was prepared on the basis of the published coordinates (PDB entry 1QL3), using the Swiss PDB Viewer/Povray program (*35*). The heme moiety is shown in red, and the lysine $\epsilon$-amino nitrogen atoms are shown in blue.

Table 2: Kinetic Parameters for the High- and Low-Affinity Phases of the Oxidation of Cytochrome $c_{552}$-f Lysine Mutants by Wild-Type Oxidase from *P. denitrificans* at an Ionic Strength of 14.8 mM

| | high-affinity | | low-affinity | |
|---|---|---|---|---|
| $c_{552}$-f | $K_M$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $K_M$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) |
| wild type | 3.2 | 136 | 18.3 | 1000 |
| K9I | 13.0 | 26 | 75 | 178 |
| K13N | 11.6 | 56 | 68 | 125 |
| K13D | 10.7 | 95 | 56 | 185 |
| K15I | 8.5 | 88 | 41 | 243 |
| K19I | 14.7 | 78 | 79 | 122 |
| K70I | 14.8 | 22 | 55 | 196 |
| K74I | 11.0 | 40 | 58 | 108 |
| K77I | 10.6 | 50 | 72 | 204 |
| K85I | 9.4 | 111 | 41 | 400 |

strength (15 mM) for which kinetics can still be determined with sufficient accuracy (see also the Discussion). The general trend, therefore, supports the view that no single mutation, not even a charge reversion in the case of the K13D mutant, has a unique effect on kinetic properties, but rather that mutations are a reflection of the general surface potential.

*Oxidase Mutants in the Docking Site on Subunit II Respond Differently to the Mitochondrial and Bacterial Cytochrome c.* Given the major difference in surface potential of the two donor molecules, the smallest bacterial $c_{552}$ fragment and the horse heart cytochrome, we analyzed the behavior of both toward mutations in the docking site on subunit II of oxidase. Table 3 lists the relevant kinetic parameters for both phases for the $c_{552}$-f donor, and Figure 4 displays the ratio of $K_M$ values (high-affinity phase) for either substrate, the $c_{552}$-f (this study) and the mitochondrial cytochrome *c* (*31*). $k_{cat}$ values for both substrates versus wild-type oxidase are almost identical, whereas the $K_M$ value for the cytochrome $c_{552}$-f oxidation is lower (3.2 $\mu$M) than for the horse heart (5.2 $\mu$M) donor.

Table 3: Cytochrome $c_{552}$-f Oxidation at an Ionic Strength of 14.8 mM by the Isolated *P. denitrificans* Four-Subunit Oxidase Complex Carrying Mutations in Specific Subunit II Residues

| | high-affinity | | low-affinity | |
|---|---|---|---|---|
| oxidase | $K_M$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $K_M$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) |
| wild type | 3.2 | 136 | 18.3 | 1000 |
| W121Q | 3.0 | 4 | 50.2 | 7 |
| N160D | 2.0 | 135 | 20.0 | 1000 |
| D146N | 5.5 | 128 | 44.5 | 270 |
| E140Q | 11.5 | 166 | 27.2 | 277 |
| H119N | 15.8 | 188 | 74.5 | 769 |
| E142Q | 10.4 | 68 | 60.2 | 154 |
| D135N | 16.0 | 39 | 38.0 | 51 |
| D178N | 14.1 | 80 | 71.7 | 333 |
| H119I/Q120I | 12.6 | 104 | 51.3 | 333 |
| TM1[a] | 23.5 | 12 | —[a] | —[a] |

[a] TM1 denotes the triple oxidase mutant (subunit II, E126Q/D135N/D178N); it exhibits monophasic behavior.

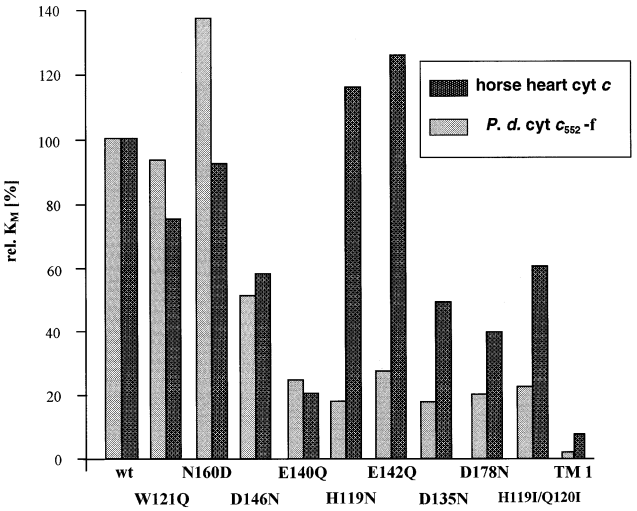


FIGURE 4: $K_M$ shifts in the reaction of docking site mutants of oxidase with two different substrates. Isolated oxidase complexes mutated in the indicated residues on subunit II were reacted with either horse heart cytochrome *c* at 56 mM or $c_{552}$-f from *P. denitrificans* at an ionic strength of 15 mM; relative binding affinity changes were plotted as the ratio of $K_M$ values (wild type vs mutant, high-affinity phase), with the wild-type oxidase values set to 100%.

In the assessment of relative $K_M$ values for both substrates, two sets of site-specific mutations in the substrate docking site on oxidase become apparent. Similar relative changes in $K_M$ are seen for mutations in E140Q, D146N, W121Q, and to some extent N160D, while a second group of mutations are characterized by large (at least 2-fold) variations in relative $K_M$ effects: H119I/Q120I, H119N, E142Q, D135N, D178N, and TM1 (see Figures 4 and 5).

## DISCUSSION

*Catalytic Proficiency of Cytochrome $c_{552}$ as the Genuine Electron Donor to the P. denitrificans Heme $aa_3$ Oxidase.* With the versatility of a typical bacterial electron transport chain, *P. denitrificans* offers several a priori possibilities for *c*-type cytochromes to mediate electron transfer between complexes III and IV (*7*, *18*). Several arguments have been put forward to suggest that a membrane-bound cytochrome, $c_{552}$, takes over this role in *Paracoccus*: its association and high electron transfer activity in a supercomplex together with the $bc_1$ complex and oxidase (*19*), and various types of immunological, genetic, and physiological evidence (*18*, *20*).

This clearly differs from the situation in mitochondria where a single soluble cytochrome *c* shuttles electrons between both complexes; its close *Paracoccus* homologue, $c_{550}$, has been probed for this function in the past in some cases (*36*, *37*), but is generally assumed to act primarily in denitrification (*38*, *39*).

By establishing kinetic parameters for the reaction of isolated wild-type oxidase with three different forms of the bacterial cytochrome $c_{552}$ differing each in length and domain complexity, we show that this bacterial donor is a kinetically fully competent donor of electrons to the $aa_3$ terminal oxidase. All three forms, the native membrane protein and two truncated variants (see Figure 1), were expressed in *E. coli* by coexpression of the *ccm* (cytochrome *c* maturation) gene cluster (*21*, *32*).

Surprisingly at first, conditions for optimal reactivity were clearly different for each of the three forms, and for the classical horse heart cytochrome *c* as a donor. The latter is characterized by its high excess of basic residues and thus its extremely high pI value, in contrast with the acidic pI of the bacterial protein (*21*). This surface charge difference easily explains the shift in the optimal ionic strength for the reaction with oxidase from 56 mM (horse heart cytochrome *c*) to 15 mM for *Paracoccus* $c_{552}$ (see Table 1). Another difference refers to the pH optimum, comparing the three forms of $c_{552}$ analyzed here. Only the shortest variant, $c_{552}$-f, whose structure has been determined recently (*22*, *23*), reacts with a high rate at pH 7.5. The larger form, $c_{552}$-ff, is a poor reductant at this condition, and the isolated native $c_{552}$ also exhibits an optimum turnover at rather acidic pH. In protein chemical terms, only the smallest fragment lacks the acidic domain (which by itself contributes a net negative charge of 8), and has previously been discussed as a potentially flexible linker region of $c_{552}$ (*20*); we assume that this domain is responsible for the pH optimum drop in the oxidase reaction for both longer variants.

Considering the $k_{cat}/K_M$ ratio (with each value taken from the relevant phase of the reaction; see above), both the native form and the smallest truncation show virtually the same catalytic efficiency, making them even better substrates than the mitochondrial cytochrome *c* (Table 1), each at their individual reaction optimum. The low $K_M$, reflecting a high binding affinity for oxidase, of the native $c_{552}$ is a reassuring argument for the genuine role of this cytochrome within the cytochrome *c* ensemble of the bacterium. At the same time, this is also surprising for the fact that electrostatic forces driving complex formation should be considerably smaller than in the mitochondrial case. In physiological terms, both the membrane association (or even more, a supercomplex connectivity) and the shift to lower ionic strength for the bacterial donor protein appear to be meaningful features, since unlike the mitochondrial situation, the bacterial periplasmic space directly relates to the outside pH and ionic strength of this soil dweller.

*Differences in Docking Sites on Oxidase between Both Donor Molecules.* Making use of several mutants in the presumed docking site of subunit II of the bacterial oxidase, we analyzed the interaction with both donors, the horse heart and the bacterial $c_{552}$-f, to map their interaction domains. The criterion used here is the $K_M$ ratio (wild type/mutant; high-affinity phase) for each of the two cytochromes. One group of oxidase mutants leads to similar effects toward both donors, while a second group shows specific, or even exclusive, effects for one or the other cytochrome *c* (see Figures 4 and 5). Remarkable $K_M$ differences relative to that of the wild type are observed for the oxidase mutations H119N and E142Q; mutants in each of these two positions are largely unaffected in the horse heart assay, but strongly diminished in their affinity when oxidizing the bacterial cytochrome (see also Figure 5). Further differences, even though less drastic, between both donors refer to positions D135 and D178, while the N160D mutant is peculiar for the fact that it shows a moderately increased affinity, possibly due to a more favorable electrostatic interaction with the bacterial donor molecule.

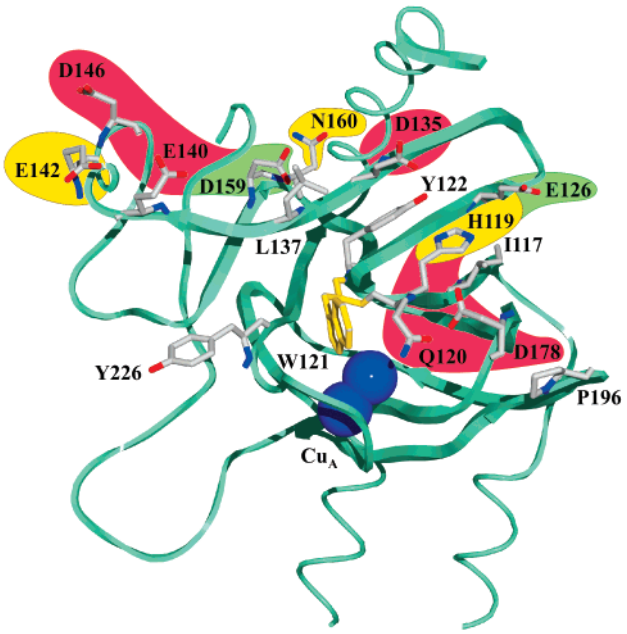


FIGURE 5: Partial structure of subunit II of the *P. denitrificans* cytochrome *c* oxidase illustrating the docking site specificity for both cytochrome *c* substrates that were characterized. Residues within the red background are involved in binding of both the horse heart cytochrome and $c_{552}$-f, while yellow background denotes those three additional residues that are reactive with only $c_{552}$-f. Quantitative differences (not specified in the figure) between both donors are also observed for positions D178 and D135. The two green colored residues have not been tested with cytochrome $c_{552}$-f. W121 with its side chain structure highlighted in yellow represents the sole residue for electron entry into oxidase. The two blue spheres depict the $Cu_A$ center metal ions.

Taken together, the above data suggest that both cytochromes, though quite different in their electrostatic properties, cover similar areas on the hydrophilic domain of subunit II. The most clear-cut effect toward both donors is obtained for the triple oxidase mutant, TM1 (*27*, *31*); removal of three carboxylate side chains causes a sharp drop in affinity (and in turnover) for both donors, yet affects interaction with the less charged docking site of the bacterial donor (see below) even more drastically (see Figure 4).

No major specific effect in behavior toward the bacterial donor is seen for the presumed site of entry for electrons into oxidase. For both substrates, W121 is the crucial residue extensively characterized earlier (*16*); when mutated, it abolishes electron flow altogether, irrespective of the donor identity, as has been observed for another bacterial oxidase as well (*17*). Interestingly, the bacterial cytochrome also leads to nonlinear kinetics, observed for many oxidases that have been tested, allowing the conclusion that biphasic reaction

behavior is not a specific property of the commonly used mitochondrial cytochrome *c* (see also ref *31*).

*Cytochrome $c_{552}$: Localized Charges or General Surface Potential?* When the 3D structure is analyzed (*22*, *23*), it is evident that despite its slight negative surplus charge at neutral pH, the hemisphere of the molecule around its heme crevice is studded with a set of up to nine lysine residues, providing a considerable positive charge density (see Figure 3 for an illustration). We mutated eight of these lysines separately in $c_{552}$-f and probed their reactivity toward isolated wild-type oxidase under standardized reaction conditions (in particular, ionic strength). To our surprise, each of the individual mutants showed clear increases in $K_M$, when compared to that of the wild-type donor. This more or less uniform (3−5-fold) loss of affinity supports the concept that not individual charges but the general surface potential drives the association reaction between both molecules (*34*). Our observation is in contrast with earlier findings from both chemical modification and site-directed mutagenesis studies with the mitochondrial cytochrome *c* (*40*, *41*), which clearly indicated dominating effects on complex stability for some of the surface-exposed lysine residues.

With no detailled structural information for the native bacterial electron donor at hand, we can only speculate about whether the soluble domain approach represents the final description of the interaction between oxidase and its donor molecule. While we here observe the interaction between a detergent-solubilized oxidase and a soluble module (or the detergent-solubilized full-size $c_{552}$) in three-dimensional space, the physiological situation in the native membrane may pose a much higher constraint on the spatial orientation of both interacting domains. The $c_{552}$ membrane anchor would fix the distance of the heme domain, and any supramolecular association (see the introductory section) with oxidase, or with oxidase and the $bc_1$ complex at the same time, would even further restrict the requirement for a diffusional encounter of both proteins.

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## SUPPORTING INFORMATION AVAILABLE

Graphs showing the dependencies of the turnover number on ionic strength and pH and two tables of kinetic parameters for cytochrome oxidation. This material is available free of charge via the Internet at http://pubs.acs.org.

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